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APPLICATION NUMBER: ***60/557,708***
FILING DATE: ***March 31, 2004***

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033104

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

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Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Takashi	Kadowaki	Kanagawa, Japan

Additional inventors are being named on the one *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Agents For Inducing Expression Of Adiponectine

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OR

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ENCLOSED APPLICATION PARTS *(check all that apply)*

[X] Specification *Number of Pages* 28
[X] Drawing(s) *Number of Sheets* 9
[ ] Application Date Sheet. See 37 CFR 1.76
[ ] CD(s), Number
[ ] Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[ ] Applicant claims small entity status. See 37 CFR 1.27.
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No.
[ ] Yes, the name of the U.S. Government agency and the Government contract number are:

[Page 1 of 2]

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Date March 31, 2004

REGISTRATION NO. 32,893 *(if appropriate)*

Docket Number: 2144.0190000/RWE

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This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Docket Number 2144.0190000/RWE

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March 31, 2004

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Commissioner for Patents
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Alexandria, VA 22313-1450

Mail Stop Provisional Patent Application

Re: U.S. Provisional Patent Application
Appl. No. (to be assigned); filed (herewith)
For: **Agents for Inducing Expression of Adiponectine**
Inventors: Kadowaki *et al.*
Our Ref: 2144.0190000/RWE

Sir:

The following documents are being submitted under 37 C.F.R. § 1.53(c) herewith for appropriate action by the U.S. Patent and Trademark Office:

1. PTO Fee Transmittal (Form PTO/SB/17);

2. U.S. Provisional Patent Application entitled:

   Agents for Inducing Expression of Adiponectine

   and naming as inventors:

   Takashi Kadowaki, Toshimasa Yamauchi, Yusuke Ito and Shoko Kitajima

   the application consisting of:

   a. A Provisional Application for Patent Cover Sheet;

   b. A specification containing twenty-eight (28) total pages:

      i. 27 pages of description prior to any claims; and

      ii. 1 page of claims (9 claims); and

c. Nine (9) sheets of drawings: (Figures 1(a-c); 2(a-c); 3(a-d); 4(a-f); 5(a-b); 6(a-g); 7(a-b); 8 and 9(a-e));

3. Authorization To Treat A Reply As Incorporating An Extension Of Time Under 37 C.F.R. § 1.136(a)(3);

4. Form PTO-2038 Credit Card Payment Form in the amount of $160.00 to cover the provisional application filing fee; and

5. Two (2) return postcards.

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Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Robert W. Esmond
Attorney for Applicants
Registration No. 32,893

RWE/dab
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Enclosures

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FEE TRANSMITTAL for FY 2004

Effective 10/01/2003. Patent fees are subject to annual revision.

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 160.00

Complete if Known	
Application Number	(to be assigned)
Filing Date	(herewith)
First Named Inventor	Takashi Kadowaki
Examiner Name	N/A
Art Unit	N/A
Attorney Docket No.	2144.0190000/RWE

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2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

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1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
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1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
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1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
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1460	130	1460	130	Petitions to the Commissioner	
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1806	180	1806	180	Submission of Information Disclosure Stmt	
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: (to be assigned)
Kadowaki *et al.*	Art Unit: N/A
Appl. No.: (to be assigned)	Examiner: N/A
Filed: (herewith)	Atty. Docket: 2144.0190000/RWE
For: **Agents for Inducing Expression of Adiponectine**	

Authorization to Treat a Reply as Incorporating an Extension of Time Under 37 C.F.R. § 1.136(a)(3)

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Mail Stop Provisional Patent Application

Sir:

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Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

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Transcription of adiponectin/Acrp30, a hormone secreted by adipocytes that acts as an antidiabetic and anti-atherogenic adipokine, is reduced in the adipose tissue in obesity, and this reduction is implicated in the development of insulin resistance in obesity. To map the promoter region of the adiponectin gene that confers this response to obesity, we made hypertrophic adipocytes model and then studied promoter activity of regions containing ~1271 base pairs (bp) to +35 bp of the promoter linked to the luciferase gene. Adipocytes transfected with plasmid containing as few as 250 bp of the adiponectin promoter had high levels of luciferase activity. However, only those cells with 436 bp of the adiponectin upstream regulatory region showed suppression of expression in hypertrophic adipocytes. Using electrophoretic mobility shift assays (EMSAs), we showed that a 32-bp fragment of DNA mapping between -344 and -313 bp upstream from the start of adiponectin expression was bound by protein factors in nuclear extracts prepared from both adipocytes and adipose tissue. There was much greater retardation of this fragment with nuclear extracts prepared from small versus large adipocytes or adipose tissue of lean versus obese mice. Then we used yeast one-hybrid strategy to identify nuclear factors that bind to this element. Among six positives from the one-hybrid screen, EMSAs and chromatin immunoprecipitation assay revealed that Kruppel-like factor 9 (KLF9) binds to this element and the amount of this binding seemed to be correlated with the KLF9 expression levels *in vitro* and *in vivo*. In addition, transient cotransfection experiments showed that overexpression of KLF9 dose-dependently and specifically enhances adiponectin promoter activity. Regulation of adiponectin transcription by KLF9 was confirmed by changes in the endogenous adiponectin messenger RNA by KLF9 siRNA and KLF9 overexpression *in vitro* and KLF9 disruption *in vivo*. Taken together, these results indicate that decreased KLF9 in obesity reduces adiponectin expression through the promoter region independent

of adipocyte-specific expression, which leads to obesity-linked insulin resistance. These data also suggest that the activators of KLF9 in adipose tissue may provide a novel treatment modality for insulin resistance and type 2 diabetes.

Introduction

Obesity is defined as an increased mass of adipose tissue and is associated with a higher risk of cardiovascular and metabolic disorders such as diabetes, hyperlipidemia, and coronary heart disease[1,2]. However, the molecular basis for this association remains to be elucidated. The adipose tissue itself serves as the site of triglyceride (TG) storage and free fatty acid (FFA)/glycerol release in response to changing energy demands[1]. It also participates in the regulation of various types of energy homeostasis as an important endocrine organ that secretes a number of biologically active substances called "adipokines" such as FFA[3], adipsin[4], tumor necrosis factor-α[5], leptin[6], plasminogen activator inhibitor-1[7], and resistin[8].

Adiponectin or Acrp30[9-12] is an adipocyte-derived hormone with multiple biological functions. Plasma adiponectin levels are decreased in obesity, insulin resistance, and type 2 diabetes[13]. Administration of adiponectin has been shown to exhibit glucose lowering effects and ameliorate insulin resistance in mice[14-16]. Conversely, adiponectin deficient mice exhibited insulin resistance and diabetes[17, 18]. This insulin sensitizing effect of adiponectin appears to be mediated by an increase in fatty acid oxidation via activation of PPARα[19,20] and also acutely via AMP kinase[21,22]. Adiponectin may have anti-atherogenic properties such as anti-inflammatory effects in human aortic endothelial cells (HAEC) and macrophages[23,24]. Increased expression of adiponectin in apoE deficient mice exhibited amelioration of atherosclerosis associated with decreased expression of the molecules involved in inflammation[19,25]. Adiponectin deficient mice displayed increased neointimal formation[17,26]. Recently, we have reported the cloning of complementary DNAs encoding adiponectin receptors (AdipoR) 1 and 2 by expression cloning[27]. AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver. AdipoR1 and R2 are predicted to contain seven transmembrane domains[27], but to be structurally and

functionally distinct from G-protein coupled receptors[28-30]. AdipoR1 and R2 serve as receptors for globular and full-length adiponectin, and mediate increased AMPK[21,22], PPARα ligands activities[19,20], and fatty-acid oxidation and glucose uptake by adiponectin[27].

The reduction of adiponectin in obesity has been implicated in the development of obesity-linked insulin resistance. We report here the molecular cloning of transcription factor, which is responsible for decreased adiponectin mRNA levels in obesity.

Results

Adiponectin mRNA levels are decreased in hypertrophic 3T3L1 adipocytes

Adiponectin mRNA levels have been reported to be decreased in obesity, which plays causal roles in the development of obesity-linked insulin resistance. The goal of this study was to isolate transcription factors that are responsible for decreased expression of adiponectin in obesity. To address this issue, we needed hypertrophic adipocyte model *in vitro* and then determined the effects of adipocyte hypertrophy on adiponectin gene expression. Interestingly, we found that 3T3L1 adipocytes 19 days after induction of adipocyte differentiation (Day 19) contained more triglyceride (Fig. 1a), exhibited insulin resistance such as decreased glucose uptake in response to insulin (Fig. 1b), expressed more insulin resistance causing adipokines such as resistin (Fig. 1c), and at the same time expressed less adiponectin mRNA (Fig. 1c) as compared with 3T3L1 adipocytes 10 days after induction of adipocyte differentiation (Day 10). Similar to the changes observed on the mRNA level, adipocyte hypertrophy decreased adiponectin protein levels (data not shown), suggesting that decreased transcription represents the mechanism underlying adipocyte hypertrophy responsiveness of the adiponectin gene.

There are signalling pathway(s) responsible for decreased expression of adiponectin in hypertrophic adipocytes other than TNFα

Previous promoter analysis of the adiponectin gene 5' flanking region identified C/EBP transcription factor(s) which confer(s) an adipocyte specific expression[31,32]. However, where the upstream region which is responsible for decreased adiponectin expression in hypertrophic adipocytes observed in obesity remains to be determined. Interestingly, the promoter activity of regions containing -1217 base pairs (bp) to +35 bp of the adiponectin promoter linked to the luciferase gene was higher in small adipocytes (Day 10) as compared with preadipocytes (Day 0) or large adipocytes (Day 19) (Fig. 2a), which was correlated with the expression levels of adiponectin.

Since TNFα, which has been shown to reduce adiponectin expression, is increased in hypertrophic adipocytes, it was reasonable to assume that TNFα could be responsible for decreased expression of adiponectin in hypertrophic adipocytes[33]. Incubation of small adipocytes (Day 10) with TNFα indeed reduced adiponectin gene promoter activity (Fig. 2b), however, neutralizing antibody against TNFα had no effect on decreased adiponectin promoter activity in hypertrophic adipocytes (Day 19) (Fig. 2c). These data suggested that there may be signalling pathway(s) responsible for decreased expression of adiponectin in hypertrophic adipocytes other than TNFα.

Adipocyte hypertrophy regulates the adiponectin promoter through a proximal 32-bp promoter element

To identify promoter regions mediating adipocyte hypertrophy responsiveness of the adiponectin gene, functional 5' deletion analysis was performed. These studies revealed that loss of the region spanning -1217 to -436 had no substantial influence on adiponectin promoter activity in 3T3L1 adipocytes at Day 19 (Fig. 3a). In contrast, removal of an additional 186 nucleotides restored adiponectin promoter activity in

3T3L1 adipocytes at Day19 (Fig. 3a), suggesting that -436/ -250 contains essential regulatory elements.

To identify the element which the regulatory transcription factor(s) was bound to, functional 5' deletion analysis was further performed using EMSA. Analysis of the -436/ -250 promoter region in EMSA studies showed that in 3T3L1 adipocytes at Day 10, one major complex was bound to this element more than as compared to 3T3L1 adipocytes at Day 19 (Fig. 3b). Loss of the region spanning -436 to -344 had no substantial influence on the amount of the binding protein in nuclear extract of 3T3L1 adipocytes at Day 10 (Fig. 3b). In contrast, removal of an additional 32 nucleotides markedly reduced it in 3T3L1 adipocytes at Day10 (Fig. 3b), suggesting that -344/ -313 comprises essential binding elements. Importantly, in obese model ob/ob mice or in 3T3L1 adipocytes at Day 19, this one major complex was bound to this 32 bp element (-344/ -313) less than as compared to lean control C57B6 mice or 3T3L1 adipocytes at Day 10, respectively (Fig. 3c).

To investigate the enhancer properties of this promoter region in detail, it was transferred to a promoter system and functionally analyzed (Fig. 3d). The presence of the -344/ -313 element conferred elevation of basal transcriptional activity (5-fold) to the per se control pGL2-tk-Luc vector in 3T3L1 adipocytes at Day 10, but not at Day 19 (Fig. 3d).

Yeast One-hybrid Cloning of the 32 bp binding protein(s)

To isolate trans-acting factors involved in the downregulation of adiponectin gene promoters during the adipocyte hypertrophy, we used the yeast one-hybrid cloning approach[34]. We used 32bp as bait and directly trimerized these sequences. We obtained 22 positive colonies. These clones were found to represent different groups. The nucleotide and deduced amino acid sequences showed that two groups, with ten

and two independent cDNA isolates, encoded a transcription factor belonging to the Kruppel like transcription factor (KLF) family[35,36], 3 and 9, respectively. One another group, with four independent cDNA isolates, encoded a transcription factor NF-κB p65[37].

EMSA revealed that the 32 bp binding complex contained KLF9 *in vitro* and *in vivo*

To further characterize the nuclear factors binding to the 32 bp, EMSA supershift experiments using specific antibodies recognizing KLF3, KLF9, or NF-κB p65 were performed. These studies identified complex I in 3T3L1 adipocytes (Day 10) as containing KLF9 protein (Fig. 4a right, lanes 1 and 2). In contrast, KLF3 (Fig.4b right) as well as NF-κB p65 (Fig. 4a right, lanes 1 and 3) were not detected in small adipocytes *in vitro*. In control EMSAs, the specificity of the KLF9 antibody was confirmed using 293T cell nuclear extracts together with a labeled KLF9 consensus site (basic transcription element:BTE) as a probe (Fig. 4c). Functionality of the KLF3 (Fig. 4b left) or NF-κB p65 antibody (Fig. 4a left) was confirmed in diminshment employing 293T expressing cognate protein nuclear extracts together with a KLF consensus site or NF-κB consensus site (p65 site) as radiolabeled probe, respectively. Importantly, the KLF9 antibody also reduced the amount of the 32 bp binding protein (Fig. 4d), suggesting that 32 bp binding protein(s) (complex) contained KLF9 *in vivo*.

To confirm these results, EMSA competition studies were carried out. We found that complex I was completely competed out by an excess of KLF consensus sequence BTE (data not shown). In contrast, NF-κB consensus sequence was clearly less effective (data not shown).

To further confirm these observations, we performed chromatin immunoprecipitation assay. We found that KLF9 indeed bound to endogenous

adiponectin promoter region containing our 32 bp site (Fig. 4e). Moreover, there was almost the same retardation of this 32 bp fragment with purified KLF9 as with nuclear extracts prepared from adipocytes or adipose tissue (Fig. 4f).

KLF9 expression increased during adipocyte differentiation, whereas decreased during adipocyte hypertrophy

We next studied the expression levels of KLF3 and 9 during adipocyte differentiation and during adipocyte hypertrophy. We found that KLF9 expression increased during adipocyte differentiation, while decreased during adipocyte hypertrophy (Fig. 5a lower). In contrast, the expression levels of KLF3 decreased during both adipocyte differentiation and hypertrophy (Fig. 5a upper). Moreover, KLF9 mRNA (Fig. 5a) and also protein levels (Fig. 5b) were higher in lean control C57BL6 mice as compared with obese ob/ob mice, which appeared to be correlated with the amounts of 32 bp binding protein, raising the possibility that the expression levels of KLF9 may regulate adiponectin promoter activity and expression levels of adiponectin.

KLF9 expression increased enhancer and adiponectin promoter activities, amounts of 32bp binding protein and adiponectin expression

To address issue, we transiently expressed KLF9 in 3T3L1 adipocytes (Day 19) (Fig. 6a-c) or in 3T3L1 adipocytes (Day 19) by using retrovirus (Fig. 6d-f) and then analyzed (Fig. 6). Overexpression of KLF9 increased the adiponectin promoter activity (-1271/+35) (Fig. 6a), 32 bp (-344/ -313) enhancer activitiy (Fig. 6c) and the amount of 32 bp binding protein (Fig. 6e) in 3T3L1 adipocytes (Day 19), demonstrating that KLF9 is able to increase adiponectin promoter activity (Fig. 6a) and the 32 bp is highly reactive to KLF9 (Fig. 6c, e). KLF9 mutant containing only its DNA-binding domain exhibited virtually no transactivating effect on the 32 bp (-344/-313) element (data not

shown), confirming the specificity of results obtained with wild type expression constructs.

To further confirm the functional importance of individual sequence within the -344/ -313 region, 32 bp (-344/ -313) mutant was characterized in transfection assays and EMSA studies (Fig. 6c and e). Compared with the wild type 32 bp (-344/ -313) element, mutation of two nucleotides within this region significantly inhibited KLF9-stimulated 32 bp enhancer activity (Fig. 6c), suggesting that KLF9 responsiveness of the 32 bp (-344/ -313) region requires the functional integrity of these elements. EMSA studies using this mutated 32 bp (-344/ -313) oligonucleotide as radiolabeled probes (Fig. 6e) revealed that this mutation had marked effect on factor binding.

Moreover, stably overexpression of KLF9 by retrovirus in 3T3L1 adipocytes (Fig. 6d) increased adiponectin expression (Fig. 6f). Taken together, these data suggested that overexpression of KLF9 in3T3L1 adipocytes (Day 19) can restore the amount of 32 bp binding protein, 32 bp enhancer activity, adiponectin promoter activity and adiponectin expression to the levels observed in 3T3L1 adipocytes (Day 10).

Suppression of KLF9 expression by siRNA decreased amounts of 32bp binding protein and adiponectin expression *in vitro*

We next investigated the functional impact of KLF9 on adiponectin expression, we tried to reduce KLF9 expression and then analysed its effects. For this purpose, we employed siRNA[38]. Suppression of KLF9 expression by siRNA (Fig. 7a) had no effect on KLF3 expression (Fig. 7a), whereas it almost completely diminished the amount of 32 bp binding protein (Fig. 7b) and at the same time greatly reduced adiponectin expression in 3T3L1 adipocytes (Day 10) (Fig. 7a). These data indicated that KLF9 was required for formation of 32 bp enhancer binding protein complex and adiponectin expression.

Disruption of KLF9 expression by gene targeting decreased amounts of 32bp binding protein and plasma adiponectin levels *in vivo*

We next examined the functional relevance of KLF9 on adiponectin expression *in vivo*, we analysed the phenotypes of KLF9 knockout mice (Fig. 8a)[36]. Interestingly, the 32 bp binding protein was not detected in nuclear extracts from WAT of KLF9 knockout mice (Fig. 8b). Importantly, plasma adiponectin levels in KLF9 knockout mice were lower as compared with control wild-type littermates, despite body weight of KLF9 knockout mice was lower as compared with control wild-type littermates (Fig. c). In contrast, there were no differences in plasma adiponectin levels between in KLF3 knockout mice and their control wild-type littermates (data not shown). These data suggested that KLF9 played an important role in the regulation of adiponectin levels *in vivo*.

Mechanisms by which adipocyte hypertrophy regulates KLF9 expression in adipocytes

We next tried to clarify the mechanisms by which adipocyte hypertrophy regulates KLF9 expression in adipocytes. KLF9 expression has been reported to be induced by thyroid hormone[36], which is known to be involved in energy expenditure. Thus we first studied the thyroid hormone receptor α expression *in vitro* and *in vivo*. Interestingly, the expression levels of thyroid hormone receptor α were decreased in 3T3L1 adipocytes (Day 19) or obese model ob/ob mice as compared with 3T3L1 adipocytes (Day 10) or lean control C57BL6 mice, respectively (Fig. 9a, b). Moreover, incubation of 3T3L1 adipocytes (Day 19) with thyroid hormone increased KLF9 expression (Fig. 9c).

We hypothesized that oxidative stress may be involved in the regulation of KLF9 expression by adipocyte hypertrophy, since KLF9 promoter has been reported to

contain AP-1site[39]. Interestingly, inhibitor of c-jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), and also together with antioxidant N-acetylcysteine (NAC), increased KLF9 (Fig. 9d) and at the same time adiponectin expression (Fig. 9e), without affecting thyroid hormone receptor α (data not shown). These data suggested that upstream mechanisms increasing KLF9 expression may consist of two pathways, at lease in part: one is thyroid hormone receptor (TR) α signalling dependent pathway and the other is independent of TR α expression but dependent on oxidative stress.

Discussion

It is thought that obesity-induced downregulation of adiponectin plays a crucial causal role in the development of obesity-linked diseases such as insulin resistance, diabetes, cardiovascular diseases. Underlying molecular determinants, however, have not yet been clarified. In the present study, we demonstrate adipocyte hypertrophy-dependent suppression of adiponectin and provide clear evidence that adipocyte hypertrophy regulates adiponectin gene expression through transcriptional mechanisms.

To provide a detailed functional analysis of molecular pathways mediating the effects of adipocyte hypertrophy on the adiponectin gene, we initially investigated participating cis- and trans-activating factors employing 5' deletion analysis of the adiponectin promoter, and compared between in the small (Day10) and large adipocytes (Day19) the activity of the luciferase constructs with different sizes of the adiponcetin promoter. Previous study identified the region responsible for reduction of adiponctin by TNFα between bp -80 and -28[33]. However, our present study clearly showed by using TNFα neutralizing antibody that TNFα was not involved in the suppression of adiponectin in response to adipocyte hypertrophy (Fig. 2b, c).

These studies revealed that a region spanning -436 to -250 is indispensable for adipocyte hypertrophy-triggered adiponectin promoter repression (Fig. 3a). EMSA revealed that in 3T3L1 adipocytes the -344/ -313 element is bound by the transcription factors (Fig. 3b, c). Adipocyte hypertrophy or obesity decreased DNA-protein complex formation at the -344/ -313 site, strongly suggesting that decreased binding to the 32 bp sequences represents an important mechanism through which adipocyte hypertrophy or obesity represses the adiponectin gene.

After identifying the proximal site at -344/ -313 as the critical promoter element mediating adipocyte hypertrophy responsiveness, we aimed to define the functional properties. For this purpose, a systematic mutational analysis of these elements was performed in functional transfection studies. In parallel, the influence of these mutations on transcription factor binding was investigated in EMSAs. Mutation of two nucleotides within this region diminished almost completely overall activity of the enhancer, and it completely ablated the binding of 32 bp oligonucleotide and transcription factors (Fig. 6c, e). These data indicate the 32 bp sequences identified as an obesity response element in the adiponectin promoter. To our knowledge, this mapping of structure/function relationships within the adiponectin promoter employing a combination of EMSA techniques and functional promoter studies represents the first detailed evaluation of KLF binding sites for transcriptional regulation of the adiponectin gene.

We then isolated cDNA encoding KLF9 from small adipocytes expression library as a 32 bp binding protein using yeast one hybrid screening[27]. Forced expression of KLF9 increased the amounts of 32 bp binding protein, 32 bp enhancer activity, adiponectin promoter activity and adiponectin expression. Conversely, suppression of KLF9 expression by specific RNAi reduced them *in vitro*. Importantly, disruption of KLF9 by gene targeting *in vivo* ablated the 32 bp binding protein and

reduced plasma adiponectin levels despite lower body weight. These data collectively strongly suggested that KLF9 is functional transcription factor, which can account for the major, if not all, portion of decreased adiponectin in hypertrophic adipocytes observed in obesity.

Transcription factor KLF9 belongs to the superfamily of kruppel-like zinc finger proteins and has been implicated in the regulation of constitutively expressed "housekeeping genes" as well as genes influencing growth and differentiation. In addition, current studies demonstrated that KLF9 also participates in the regulation of inducible gene expression and that interaction of KLF9 with other transcription factors and/or cofactors such as CREB-binding protein, p300, or CtBP may represent an important transcriptional control mechanism.

Adipocyte hypertrophy observed in obesity responses comprise various signaling cascades including the stress-related MAPK-JNK as well as pathways regulating energy expenditure such as thyroid hormone. Our current study shows that JNK inhibitor or together with antioxidant NAC or thyroid hormone treatment of 3T3L1 adipocytes (Day 19) led to increased expression of KLF9 and adiponectin, indicating oxidative stress/JNK activation and decreased thyroid hormone/TRα signalling may be involved in the downregulation of adiponectin observed in hypertrophic adipocytes (Fig. 9).

In conclusion, KLF9 encodes functional transcription factor whose reduction is involved in adipocyte-hypertrophy induced hypoadiponectinemia. Molecular identification of KLF9 should facilitate the understanding of molecular mechanisms of donwregulation of adiponectin/Acrp30 in obesity and obesity-linked diseases such as diabetes and atherosclerosis and the designing of novel antidiabetic and anti-atherogenic drugs with KLF9 as molecular targets.

Methods

Materials and general methods. The 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone (DEX), NAC and SP600125 were purchased from Sigma Co. All other materials were from the sources given in the References (27, 35 and 36). DNA sequencing was performed with the PRISM dye terminator cycle sequencing kit and an ABI PRISM 310 genetic analyzer (Applied Biosystems).

Animals and blood sample assays. KLF9 deficient mice have been described elsewhere[36]. Fifteen-week-old ob/ob mice and their wild-type C57BL/6 mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). Mice were housed in colony cages, maintained on a 12-h light/12-h dark cycle. The animal care and procedures were approved by the Animal Care Committee of the University of Tokyo. Plasma glucose levels were determined using glucose B-test (Wako Pure Chemical Industries, Osaka, Japan). Plasma adiponectin levels were determined by mouse adiponectin radioimmunoassay (RIA) kit (LINCO Research Inc.).

The cDNA library The primary cDNA library was prepared from fully differentiated 3T3-L1 adipocytes (constructed in the pGAD-GH GAL4 vector)[40]. It contained 10,000,000 individual transformants, all with a cDNA insert of the size between 1.5-3 kb is kindly provided by A.Saltiel.

One-hybrid Cloning in Yeast. General methods for one-hybrid cloning and related experimental manipulations in yeast were as described[34]. We constructed a yeast strain derivative of YM4271 (Clontech). In this strain, we integrated a HIS3 reporter gene construct ((G4HSE I)x3::HIS3) containing a trimer of the 32bp sequences from adiponectin promoter (from positions -344 to -313, see Fig. 3). This construct was obtained from plasmid pHISi (Clontech) by insertion, between the XbaI (end-filled with Klenow DNA polymerase) and EcoRI sites, of the DNA fragment generated by

annealing of the following complementary oligonucleotides: 5'-GAAGCCCAAGCTGGGTTGTACCAGGTTCCCTA-3' (top strand).

For one-hybrid screening, the (32 bp)x3::HIS3 reporter yeast strain was transformed with DNA prepared from the embryo cDNA library, after amplification of 1,660,000 primary clones. Five million yeast transformants were plated on SD-His-Leu + 15 mM 3-aminotriazole. After 4-8 days of growth at 30 ° C, 22 putative positive yeast clones were selected for further analyses. Two cDNAs encoded the same KLF9 and are described in this work.

Luciferase assays. Luciferase assays were carried out with cells plated on 12-well plates as described previously[27,35]. The indicated amount of each expression plasmid was transfected simultaneously with a luciferase reporter plasmid (0.25 μg) and pSV- β gal (0.1 to 0.4 μg). The total amount of DNA in each transfection was adjusted to 1.5 μg/well with control vector DNA. The amount of luciferase activity in transfectants was measured and normalized to the amount of β -galactosidase activity as measured by standard kits (Promega).

Gel mobility shift assays. Gel mobility shift assays were done as previously described[34]. Briefly, nuclear extracts were prepared from 293T cells or 3T3L1 adipocytes or white adipose tissue as described previously[34]. Double-stranded oligonucleotides used in gel mobility shift assays were prepared by annealing both strands. The labeled probes (3,000 to 10,000 cpm) were incubated with nuclear extracts (3 μg) in a mixture (20 μl) containing 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 0.05 mM EDTA, 2.5 mM MgCl2, 8.5% glycerol, 1 mM dithiothreitol, poly(dI-dC) at 0.5 μg/ml, 0.1% Triton X-100, and nonfat milk at 1 mg/ml for 30 min on ice. The DNA-protein complexes were resolved on a 4.6% polyacrylamide gel at 140 V for 1 h at 4° C. Gels were dried and exposed to a BAS2000 filter with BAStation software (Fuji Photo Film Co., Ltd.). For competition experiments, at least a 100-fold molar excess of unlabeled

DNA relative to labeled DNA was added to the reaction mixture before addition of the labeled probe. In the supershift experiments, the gel shift reactions were first incubated for 1 h with 2 to 10 μg of polyclonal antibodies against KLF9 or KLF3 or NF-κB p65 on ice.

Retrovirus production and infection. 10^7 Plat-E packaging cells[41] were transiently transfected with 10 μg of mouse KLF9 using Lipofectamine PLUS (Life Technologies). and supernatants (10 ml) were harvested after 24 h of incubation. 3T3L1 adipocytes were infected with 1/20-diluted supernatants supplemented with 10 μg/ml polybrene (hexadimethrine bromide, Sigma) corresponding to an estimated m.o.i. of 0.3.

Plasmids. Luciferase gene constructs containing 1271-bp, 436-bp and 250-bp fragments of the adiponectin promoter (pAdiponectin1271-Luc, pAdiponectin436-Luc and pAdiponectin250-Luc, respectively) were subcloned into the pGL2 basic or pGL2 promoter vector (Promega).

Expression in mammalian cells. The KLF3 or KLF9 expression vector was constructed by ligating into the *ECo*RV/*Not*I site of pCDNA3.1. DNA transfection was performed by lipofection using Lipofectamine Plus (Gibco BRL) for 293T and 3T3L1 adipocytes.

Studies with 3T3L1 cells 3T3L1 cells were cultured in DMEM with 10% fetal calf serum, and the induction of adipogenic differentiation was carried out according to a method described previously[42]. In brief, cells were cultured and propagated to confluence. Two days later, the medium was replaced with standard differentiation induction medium containing 0.5mM IBMX, 1 μM DEX, 5 μg/ml insulin, 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin, and the medium was renewed every other day. Glucose uptake was determined as previously described[21]. Cell lysates were extracted, and their TG content was determined as described previously[15].

RNA interference. Two pairs of siRNAs were chemically synthesized, annealed, and transfected into 60%-70% confluent 3T3L1 adipocytes using Lipofectamine PLUS (Life Technologies)[27]. The sequences of the siRNAs KLF9 and KLF3 corresponded to 399-419, -92- -72, 315-335, 849-869 from the start codon of the respective cDNA. Forty-eight hours after transfection, the cells were lysed.

Northern blot analysis and quantitative analysis of transcripts by real-time PCR Total RNA was prepared from cells or tissues with TRIzol (GIBCO/BRL) according to the manufacturer's instructions. For northern blot analysis, equal aliquots of total RNA in each group were pooled (total, 10 μg), subjected to formalin-denatured agarose electrophoresis, and transferred to nylon membrane (Hybond N; Amersham Pharmacia Biotech). The filters were hybridized with [^{32}P]dCTP-labeled cDNA probe corresponding to the mouse KLF9 and mouse KLF3 cDNA, respectively. The resulting bands were visualized by exposure to BAS2000 filters with BAStation software (Fuji Photo Film Co., Ltd.). For quantification of mRNAs, we employed the real-time PCR method[27]. The primer sets and the probes were designed using Primer Express 1.5a software and purchased from ABI (ABI Prism; Perkin-Elmer Applied Biosystems, Foster City, California, USA). The relative amount was normalized to the amount of actin transcript in the same cDNAs[27].

Nuclear extract preparation and immunoblot analysis. Nuclear extracts were prepared as described previously[34]. The samples of 30 μg of nuclear protein were subjected to immunoblot analysis with rabbit immunoglobulin G (IgG) against KLF9[43] or KLF3[44], followed by horseradish peroxidase-linked mouse or rabbit IgG and the ECL kit (Amersham Pharmacia Biotech).

Chromatin immunoprecipitation assays. Quiescent 3T3L1 adipocytes fixed in 1% formaldehyde. The fixed chromatin samples were subjected to immunoprecipitation as

described[35], with minor modifications. Protein A (Upstate) was used to preclear samples and for immunoprecipitation.

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Figure Legends

Figure 1 Adipocyte hypertrophy reduces adiponectin mRNA levels in 3T3L1 adipocytes. Triglyceride content (TG)(a), glucose uptake (b) and mRNA levels of adiponectin and resistin (c) in 3T3L1 cells during adipocyte differentiation and hypertophy. TaqMan real time reverse transcription-PCR analysis of 3T3L1 adipocytes during adipocyte differentiation and hypertrophy (c). Given are the expression levels of adiponectin or resistin versus 36B4 mRNA levels in multiples of the basal level. Data shown represent a typical result obtained from a series of three independent experiments.

Figure 2 There are signalling pathway(s) responsible for decreased adiponectin promoter activity in hypertrophic adipocytes other than TNFα. Adiponectin promoter activity in 3T3L1 cells during adipocyte differentiation and hypertrophy (a), treated with or without indicated concentrations (ng/ml) of TNFα 10 days after induction (b) or treated with or without 3 ng/ml TNFα incubated with or without the indicated concentrations (μg/ml) of anti-TNFα antibody indicated days after induction (c). 3T3L1 cells were transiently transfected with adiponectin promoter (-1271/+35)-Luc. The results are expressed as the ratio of the value of control vector (a) or vehicle-treated control (b, c). Each bar represents the mean ± SE (n = 5-7). (*, $P < 0.05$; **, $P < 0.01$; compared with untreated cells).

Figure 3 Adipocyte hypertrophy regulates the adiponectin promoter through a proximal 32-bp promoter element. a, 3T3L1 adipocytes were transiently transfected with adiponectin promoter 5' deletion constructs as

indicated, 10 or 19 days after induction (Day 10 or Day 19, respectively), and assayed for luciferase activity. **b,** Nuclear protein extracts from 3T3L1 adipocytes (Day 10 or Day 19) were prepared and subjected to EMSA analysis using the indicated adiponectin promoter 5' deletion sequences as 32P-labeled probe. Data shown represent a typical result obtained from a series of three independent experiments. **c,** Nuclear protein extracts from 3T3L1 adipocytes (Day 10 or Day 19) or WAT from lean control C57 or obese model ob/ob mice were prepared and subjected to EMSA analysis using the adiponectin promoter (-344/ -313) sequence as 32P-labeled probe. **d,** The -344/ -313 fragment was transferred into the enhancerless vector pGL2-tk-Luc and transfected into 3T3L1 adipocytes (Day 10 and Day 19), and then luceferase activities were assayed. The results are expressed as the ratio of the value of control vector (a, d). Results are expressed as mean ± S.E. of three separate experiments (asterisks indicate statistically significant differences; *; $p < 0.05$). Data shown represent a typical result obtained from a series of three independent experiments (b, c).

Figure 4 EMSA revealed that the 32 bp binding complex contained KLF9 *in vitro* and *in vivo*. a-d, f, Nuclear extracts from3T3L1 adipocytes (Day 10) (a, b) or 293T (a-c) or WAT from lean control C57BL6 (B6) or obese model ob/ob mice (d) or purified FLAG-tag KLF9 (f) and radiolabeled adiponectin promoter (-344/ -313) probe (a, b, d, f) or radiolabeled NF-κB consensus sequence (p65 site) (a) KLF consensus sequence (BTE) (b, c) were incubated with or without specific antibodies recognizing KLF9 or NF-κB p65 (a, c,d), KLF3 (b). The arrows indicate specific complexes. **e,** Chromatin immunoprecipitation assay of KLF9 binding to the endogenous adiponectin promoter in 3T3L1 adipocytes (Day10). Data shown represent a typical result obtained from a series of three independent experiments.

Figure 5 KLF9 expression increased during adipocyte differentiation, whereas decreased during adipocyte hypertrophy. **a, b,** The amounts of KLF3 **(a, upper)** and KLF9 mRNA **(a, lower)** and mKLF9 protein **(b)** in 3T3L1 adipocytes indicated days after induction **(a, b)** or WAT from lean control C57B6L or obese model ob/ob mice **(a-c)**. Each bar represents the mean±s.e. (n=3-5). (*, $P < 0.05$; **, $P < 0.01$; between the two groups indicated, or compared with untreated cells).

Figure 6. KLF9 expression increased enhancer and adiponectin promoter activities, amounts of 32bp binding protein and adiponectin expression. **a-d, f,** Adiponectin promoter activity ((-1271/+35)-Luc) in 3T3L1 adipocytes (Day19) (a) or wild-type or mutated 32 bp-tk-Luc activity (c) or the amounts of KLF9 (b, d) or adiponectin mRNA (f) in 3T3L1 adipocytes (Day 19) transiently (a, b) or retrovirally (c,d, f) transfected with or without KLF9. **e,** Nuclear protein extracts from 3T3L1 adipocytes (Day 19) retrovirally transfected with KLF9 were prepared and subjected to EMSA analysis using the adiponectin promoter (-344/ -313) sequence as 32P-labeled probe. The results are expressed as the ratio of the value of control vector (a) or vehicle-treated control (b, c). Each bar represents the mean ± SE (n = 5-7). (*, P < 0.05; **, P < 0.01 ; compared with untreated cells).

Figure 7. Suppression of KLF9 expression by siRNA decreased amounts of 32bp binding protein and adiponectin expression *in vitro*. **a,** The amounts of KLF3, KLF9 and adiponectin mRNA in 3T3L1 adipocytes (Day 10) transfected with the indicated siRNA duplex. **b,** Nuclear protein extracts from 3T3L1 adipocytes (Day 10) transfected with the indicated siRNA duplex were prepared and subjected to EMSA analysis using the adiponectin promoter (-

344/ -313) sequence as 32P-labeled probe. The results are expressed as the ratio of the value of control vector (a) or vehicle-treated control (b, c). Each bar represents the mean ± SE (n = 5-7). (*, P < 0.05; **, P < 0.01 ; compared with untreated cells).

Figure 8. Disruption of KLF9 by gene targeting ablated 32bp binding protein and decreased plasma adiponectin levels *in vivo*. **a, c,** The amounts of KLF9 mRNA in WAT (a) or plasma adiponectin and body weight (c) of KLF9 deficient mice or their control wild-type littermates. **b,** Nuclear protein extracts from WAT of KLF9 deficient mice or their control wild-type littermates were prepared and subjected to EMSA analysis using the KLF9 consensus sequence (BTE) or adiponectin promoter (-344/ -313) sequence as 32P-labeled probe. The results are expressed as the ratio of the value of control WT (a).

Figure 9 Mechanisms by which adipocyte hypertrophy regulates KLF9 expression in adipocytes. **a, b,** The amounts of TRα (a, b), KLF9 (c, d) and adiponectin mRNA (e) in 3T3L1 adipocytes (Day 10 or Day 19) treated with or without indicated concentrations of T3 (**c**) or the antioxidant N-acetylcysteine (NAC)(20 mM) or JNK inhibitor SP600125 (**d, e**) or in WAT from lean control C57BL6 (B6) or obese model ob/ob mice (b). Each bar represents the mean±s.e. (n=3-5). (*, P < 0.05; **, P < 0.01 ; between the two groups indicated, or compared with untreated cells).

1．以下（A）から（D）のいずれかを有効成分とするアディポネクチン発現誘導剤。

(A) KLF 9 (Kruppel-Like factor 9)

(B) KLF9 をコードした遺伝子

(C) KLF9 をコードした遺伝子を保持した発現ベクター

(D) アディポネクチンプロモータ領域中-344 から-313b p の 32bp の核酸に結合し得る物質

2．請求項１記載のアディポネクチン発現誘導剤を有効成分とするインスリン抵抗性改善剤。

3．請求項１記載のアディポネクチン発現誘導剤を有効成分とする２型糖尿病の予防及び又は治療剤。

4．請求項１記載のアディポネクチン発現誘導剤を有効成分とする動脈硬化の予防及び又は治療剤。

5．以下の工程を含むアディポネクチン発現誘導剤をスクリーニングする方法。

(A) アディポネクチンプロモータ領域中-344 から-313b p の 32bp の核酸と被検物質を接触させる工程、

(B) 前記核酸と結合した被検物質を検出する工程。

6．アディポネクチンを発現し得る細胞に（B）において検出された被検物質を作用させ、アヂポネクチンの発現が上昇した被検物質を選択する工程を更に含む、請求項５記載のスクリーニング方法。

7．アディポネクチンプロモータの下流にリポータ遺伝子が接続された発現カセットを保持した細胞に、B）において検出された被検物質を作用させ、アヂポネクチンの発現が上昇した被検物質を選択する工程を更に含む、請求項５記載のスクリーニング方法。

8．被検者由来の試料を用いてアディポネクチンプロモータ領域または KLF9 の遺伝子配列を検査する工程を含む、イシスリン抵抗性、２型糖尿病または動脈硬化症の素因を検査する方法。

9．アディポネクチンプロモータ領域の検査として、アディポネクチンプロモータ領域の-344 から-313b p の配列を検査する、請求項８記載の検査方法。

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Fig1

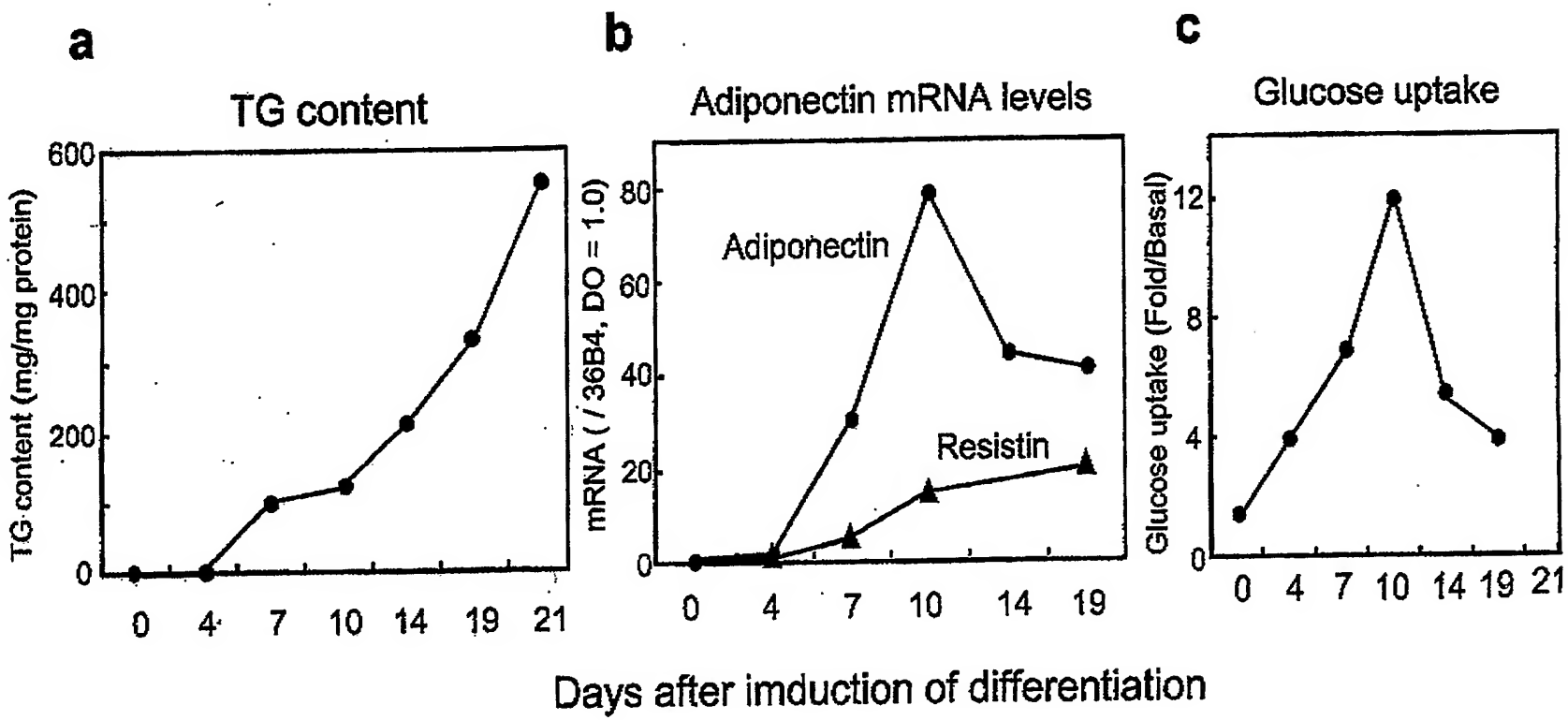
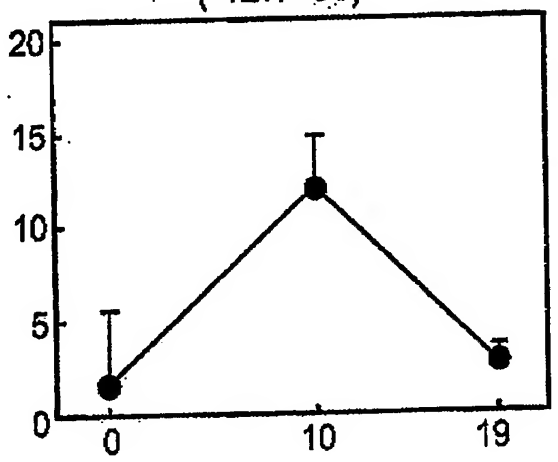
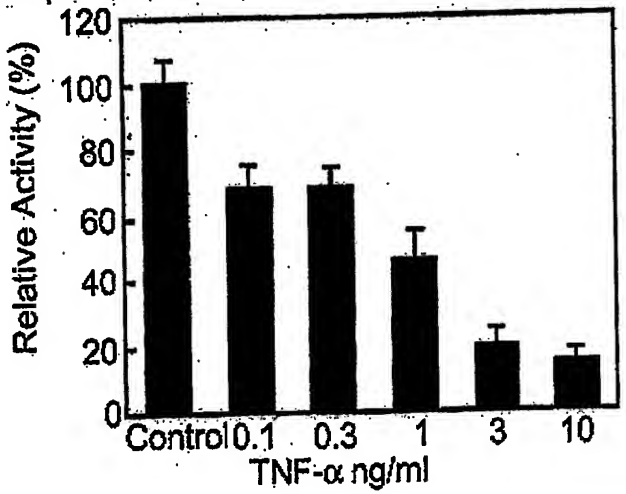


Fig2

a Adiponectin promoter activity (-127/+35)



b Effects of TNF- α on the promoter activity of adiponectin in 3T3-L1 adipocytes (Day10)



c Effects of anti-TNF-α αντιβοδψ on the promoter activity of adiponectin in 3T3-L1 adipocytes

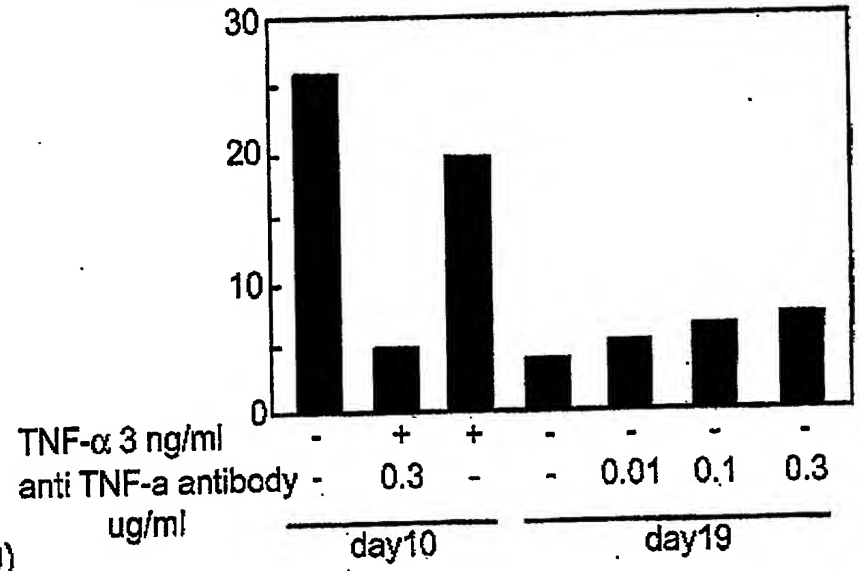


Fig3

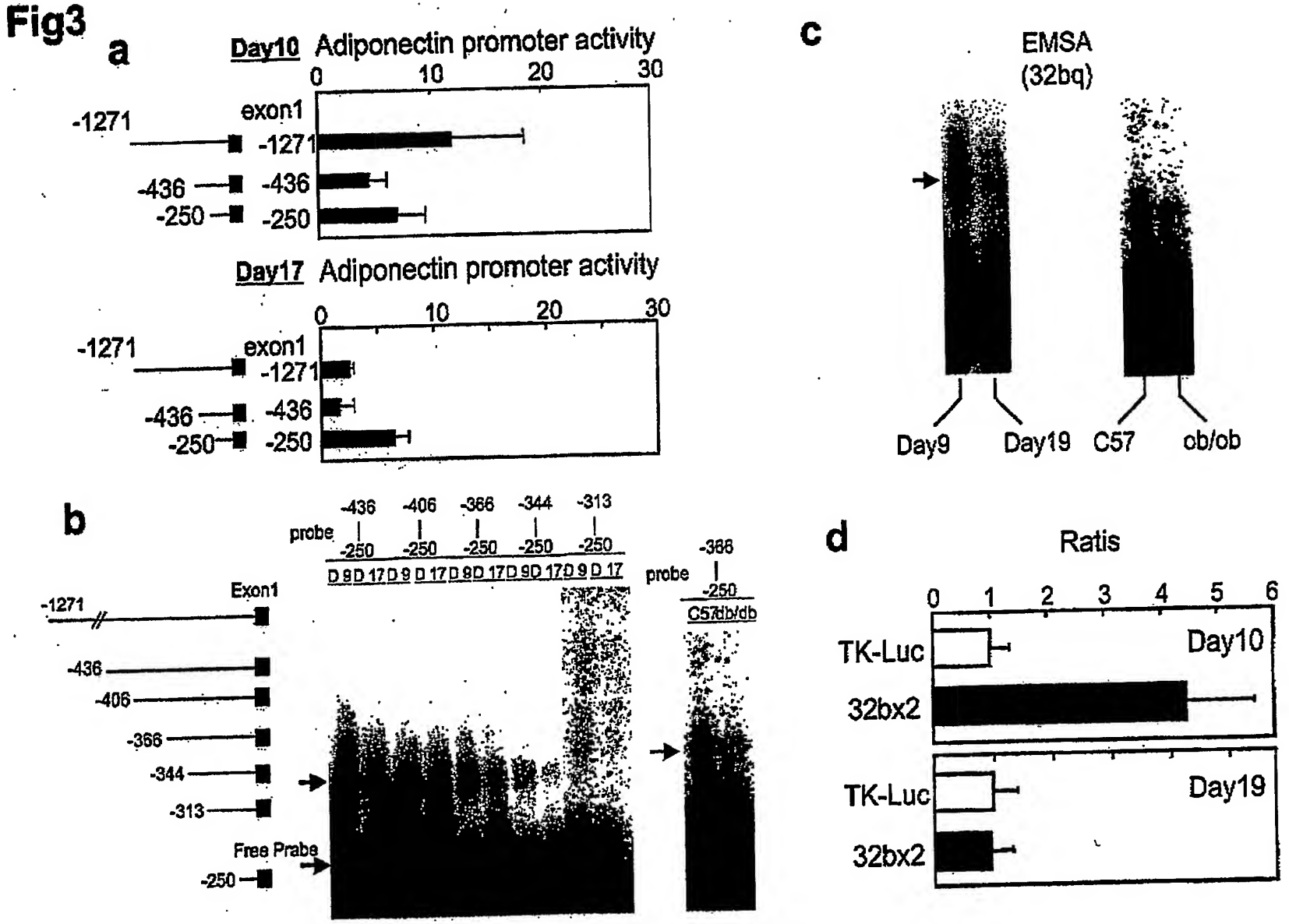


Fig4

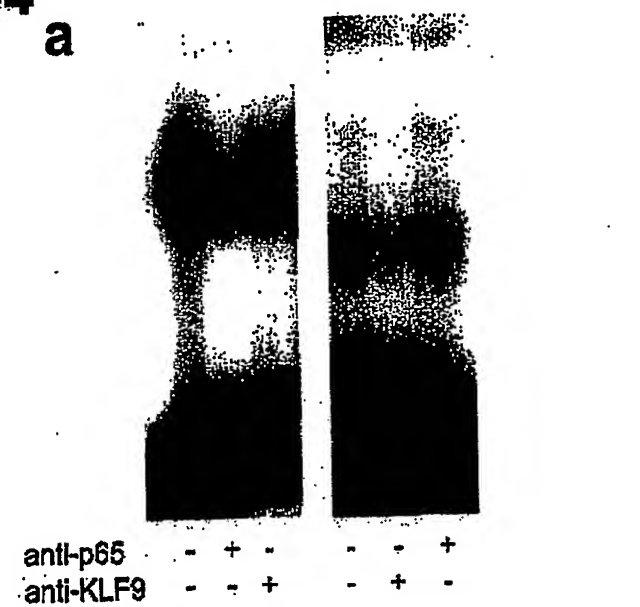
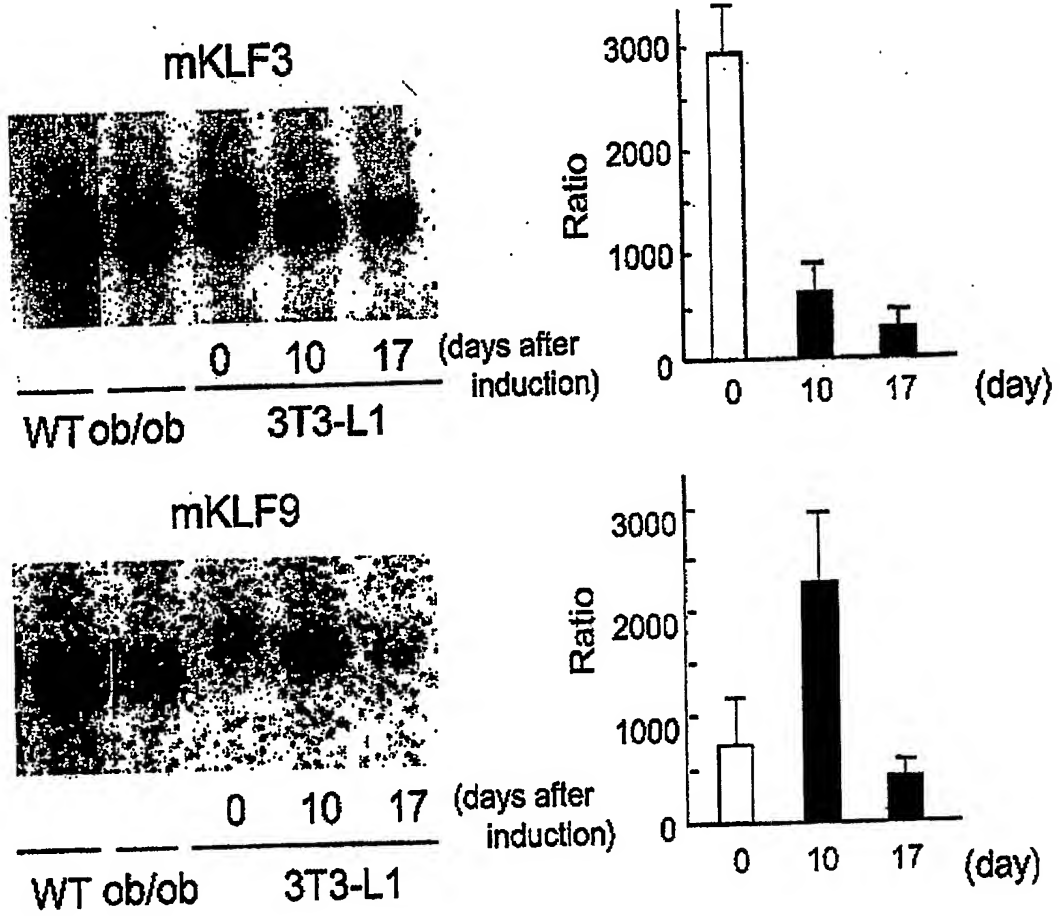


Fig5

a



b

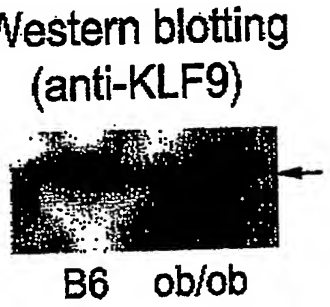


Fig6

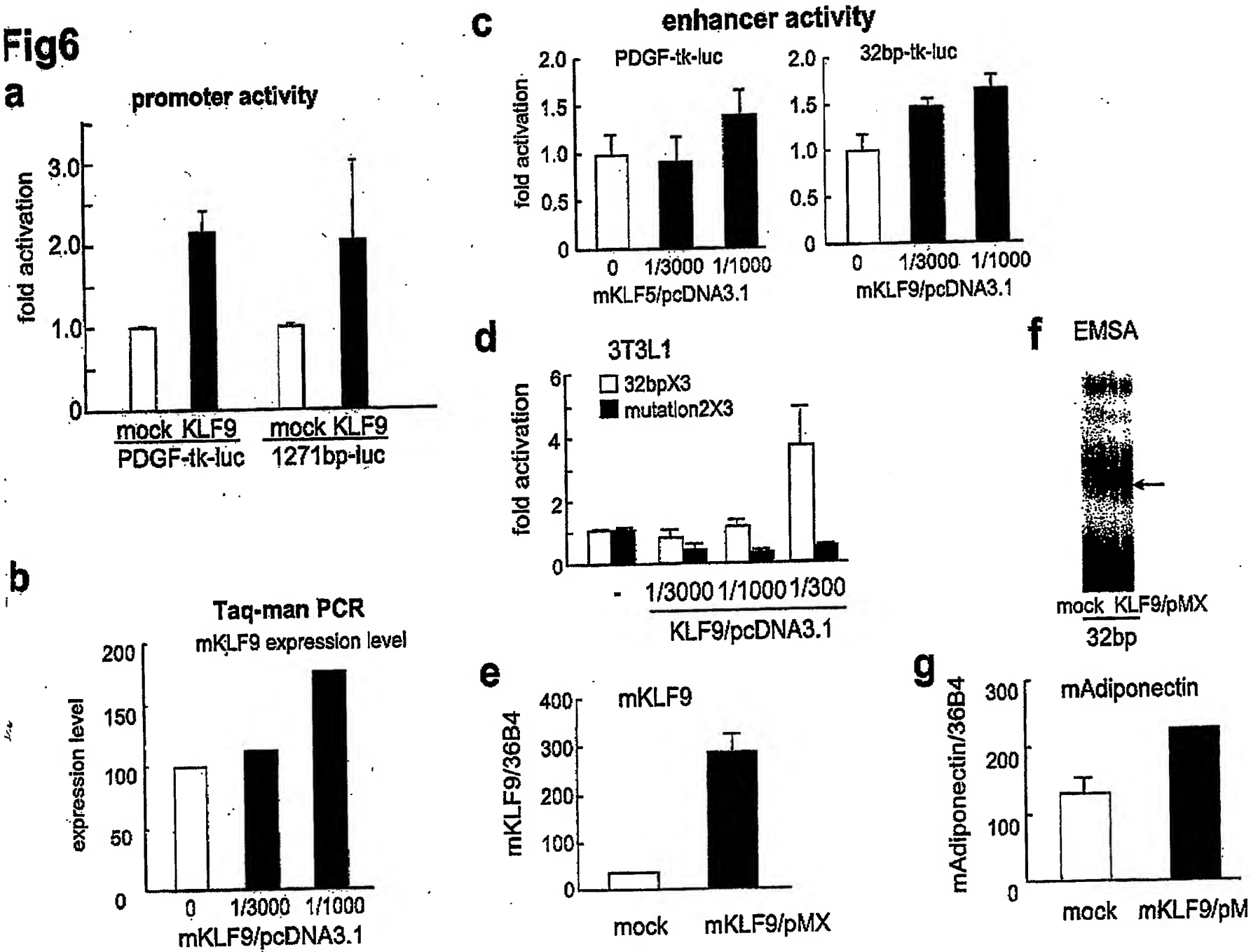
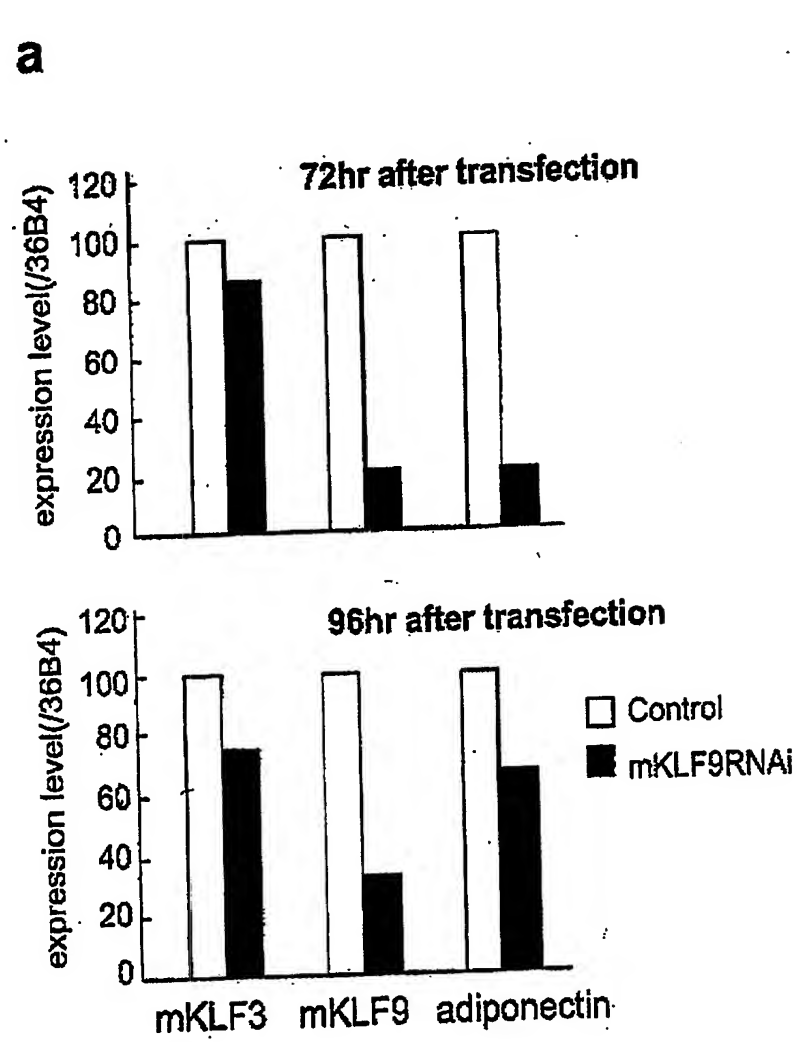


Fig7



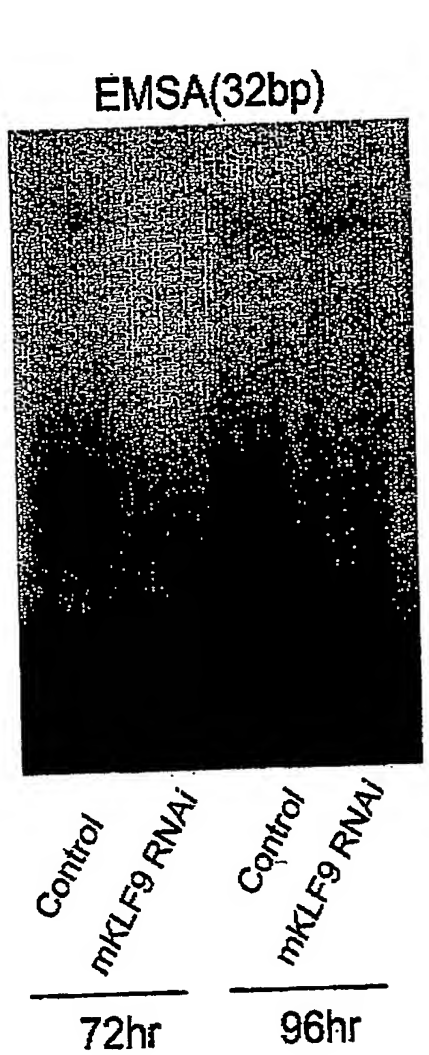


Fig8

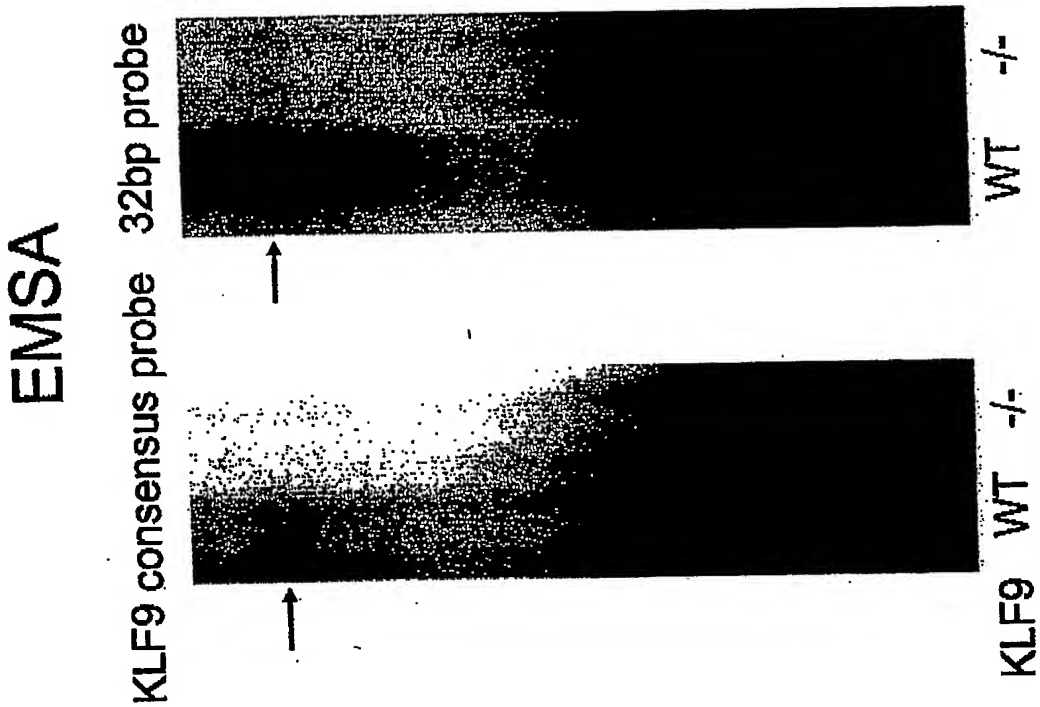


Fig9